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Human Herpesvirus-6 and Human Herpesvirus-7 Infections in Bone Marrow Transplant Recipients

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Human cytomegalovirus (HCMV), human herpesvirus-6 (HHV-6), and human herpesvirus-7 (HHV-7) DNA in peripheral blood leukocytes (PBL) of 61 bone marrow transplant recipients was monitored weekly during the first 12 weeks post-transplantation by a nested polymerase chain reaction (PCR). Thirty-seven (61%), 17 (28%), and 32 (53%) of patients had one or more PBL specimens positive for HCMV, HHV-6 or HHV-7 DNA, respectively. HHV-7 DNA in PBL during the early post-transplant period was associated with a longer time to neutrophil engraftment (mean 28.8 days vs 19.8 days; P = 0.01). In two patients who failed to engraft, HHV-6 DNA and HHV-7 DNA was detected in plasma and PBL, respectively, early in their post-transplant period. Patients with HCMV disease were more likely to have concurrent HHV-7 DNA in PBL prior to onset of disease than were patients with asymptomatic HCMV infection, suggesting that HHV-7 may be a cofactor in the progression from HCMV infection to HCMV disease. In the 17 patients (179 specimens) in whom viral DNA in plasma was studied (in addition to PBL), a positive result was found only in 3. In each, viral DNA in plasma appeared to correlate with clinically significant disease. HHV-7 DNA in plasma was associated with encephalitis in an allograft recipient. ***J. Med. Virol. 53:295–305, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Human herpesvirus-6 (HHV-6) was first isolated by Salahuddin et al. [1986] and subsequently shown to be ubiquitous in all parts of the world by seroepidemiological surveys [Briggs et al., 1988; Kangro et al., 1994]. Infection is usually acquired in infancy and 60–95% of adults are seropositive to the virus. The $CD4^+$ T cells are the main site of active viral replication, but the cellular tropism appears to be wide and includes $CD8^+$ T cells, natural killer (NK) cells, macrophages, and probably even epithelial cells [Downing et al., 1987].

The etiologic role of HHV-6 in exanthem subitum (roseola infantum) has been well defined [Yamanishi et al., 1988], and there is good evidence to suggest that primary HHV-6 infection is a common cause of acute nonspecific febrile illness in infants and children [Pruksananonda et al., 1992]. Association of HHV-6 infection with interstitial pneumonitis [Carrigan et al., 1991], fatal encephalitis [Drobyski et al., 1994], marrow suppression [Drobyski et al., 1993a], and acute graft-versus-host disease (GVHD) [Appleton et al., 1995] in bone marrow transplant (BMT) recipients has been suggested. However, establishing a pathogenic role for the ubiquitous virus has proved difficult [Kadakia et al., 1996].

Human herpesvirus-7 (HHV-7) was first isolated in 1989 from the peripheral blood lymphocytes (PBL) of a healthy individual [Frenkel et al., 1990]. Primary infection commonly occurs in childhood and approximately 90% of the healthy adult population is seropositive. More than 75% of them shed HHV-7 in their saliva [Wyatt et al., 1991, 1992]. Activated $CD4^+$ T cells are susceptible to HHV-7 infection in vitro. Primary HHV-7 infection has been reported to be associated with rash [Asano et al., 1995], exanthem subitum [Tanaka et al., 1994] and a chronic active Epstein-Barr virus (EBV) infection-like syndrome [Kawa-Ha et al., 1993], but these results await independent confirma-

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tion. Relatively little information is available in regard to HHV-7 and immunocompromised patients [Osman et al., 1996; Wang et al., 1996].

All human herpesviruses known to date are important pathogens in immunocompromised hosts, human cytomegalovirus (HCMV) being associated with the greatest morbidity and mortality. HHV-6 and HHV-7 are β-herpesviruses closely related to HCMV [Lawrence et al., 1990]. The lymphotrophic nature of HHV-6 and HHV-7; the in vitro observations that these viruses affect expression of interferon (IFN), interleukin (IL)-8, IL-1β, and tumor necrosis factor-α (TNF-α) [Kikuta et al., 1990; Inagi et al., 1996; Flamand et al., 1991]; and their ability to modulate other immunologically important cell surface molecules (CD3, CD4, and EB1) [Furukawa et al., 1994; Hasegawa et al., 1994] suggest that these viruses may have immunomodulatory potential. They may possibly play a role as cofactors of other infections [Osman et al., 1996] or in the pathogenesis of GVHD [Appleton et al., 1995]. For these reasons, it was felt worthwhile to study these viruses in BMT patients and their interactions with HCMV in particular.

The results of a longitudinal study on the activities of the three human herpesviruses (HCMV, HHV-6, and HHV-7) during the early post-BMT period are presented defining the profile of virus activity, clinical correlation with disease, and the effects of antiviral agents on HHV-6 and HHV-7 DNA in PBL.

MATERIALS AND METHODS

Patients

A total of 61 consecutive patients undergoing BMT at the Queen Mary Hospital in Hong Kong were enrolled in a prospective study. Thirty-four of the recipients were female and 27 were male, with a median age of 33 years (range: 3–50). Twenty-two had acute myeloid leukemia (AML), 17 had chronic myeloid leukemia (CML), and 22 received transplants for other diseases. Of the 61 transplants, 10 were autologous, 1 was syngeneic, 43 were allogeneic from matched related donors (allo-MRD), and 7 allogeneic from matched unrelated donors (allo-MUD). Polymerase chain reaction (PCR) for HCMV DNA on PBL was performed weekly as part of routine surveillance, these results being used for clinical management. Results of the HHV-6 and HHV-7 PCRs were available only in retrospect and did not influence diagnosis or patient management.

Specimen Collection

Ten ml of clotted blood was collected from each BMT donor and recipient pre-transplantation. HCMV serostatus was determined on all donors and recipients. In addition, the last 17 of the 61 patients also had HHV-6 and HHV-7 serostatus determined.

Ten ml of heparinized blood was collected from each marrow graft recipient at weekly intervals during the first 90 days post-transplantation. PBL were separated by sedimentation with 6% dextran in phosphate-buffered saline (PBS). The residual erythrocytes in the cell pellet were lysed with two cycles of hypotonic saline at 4°C for 3 min. The PBL were resuspended at $5–10 \times 10^5$ cells/ml in double distilled water and boiled for 15 min. Cell debris in the PBL lysate was spun down and the supernatant collected for PCR assays for viral DNA.

For the 17 patients enrolled in the latter 7 months of the study, 1 ml of cell-free plasma was collected from the same heparinized blood samples and stored frozen at −70°C for viral DNA detection in plasma, as well as PBL.

All specimens from the BMT recipients and donors used in this study were collected for surveillance for HCMV infection, which was a part of the routine patient management protocol. Blood samples were collected from 30 healthy controls after informed and written consent. PBL were separated from the heparinized blood samples processed as above. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Hong Kong.

PCR Amplification and Detection of Amplified DNA

The recommendations of Kwok and Higuchi [1989] were followed strictly to avoid contamination of the PCR samples. A negative control was run following each fifth sample. Two positive controls equivalent to 50 and 5 copies of the corresponding DNA were run in all HHV-6 and HHV-7 PCR assays and positive control equivalent to 100 genome copies was used in all HCMV PCR assays. Assays with suboptimal sensitivity of the positive controls were repeated. When consecutive samples in a PCR run were positive, they were repeated in separate assays to exclude cross-contamination.

Seventy samples that were negative for HCMV, HHV-6, and HHV-7 DNA were randomly selected and spiked with an equivalent of 300 genome copies of either HHV-6 or HHV-7 DNA and PCR was repeated to confirm the absence of inhibitors.

HCMV DNA was detected using a nested PCR as described previously [Yuen et al., 1995]. HHV-6 DNA was detected by a nested PCR amplifying a consensus sequence of the major capsid protein encoding region for both HHV-6 variants A and B [Secchiero et al., 1995a]. Five μl of sample ($2.5–5 \times 10^3$ cells) was amplified in a 50-μl reaction mixture containing PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl and 1.5 mM $MgCl_2$), 200 μM deoxynucleoside triphosphates, 1.25 units *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) and 0.125 μM each external primer [Secchiero et al., 1995a]. An initial denaturation at 92°C for 4 min was followed by 35 cycles of 1 min each at 92°, 50°, and 72°C respectively, with 10 minutes of final extension at 72°C after the last cycle of amplification. One microliter of the product from the first PCR was amplified in a second PCR with the same conditions, except 0.25 μM of internal primers [Secchiero et al., 1995a] were used.

HHV-6 variant A has a 228-bp deletion in the imme-

diate–early region. A PCR that amplifies this region was used to type the HHV-6-positive specimens as variant A or B as described previously [Yalcin et al., 1994] with the following modifications. Twenty μl of sample was amplified in a 100-μl PCR-mix containing 2.5 units *Taq* polymerase and 0.25 μM each of the variant typing external primers. After an initial denaturation step of 94°C for 3 min, there followed 30 cycles of amplification of 90°C for 1 min, 62°C for 2 min and 72°C for 3 min. The final extension step was 7 min at 72°C. Five μl of the first round product was used as template for a second PCR carried out under the same conditions, except that 0.25-μM internal primers were used instead.

HHV-7 DNA was detected by a nested PCR with external and internal primers as described previously [Wilborn et al., 1995]. Five μl of sample were amplified in a 50-μl PCR mix containing 1.25 units *Taq* polymerase, and 0.125 μM of each external primer. An initial denaturation at 94°C for 3 min was followed by 40 cycles of 94°, 54°, and 72°C for 1 min each and 5 min of extension at 72°C after the last cycle. One μl of the first PCR product was re-amplified in a 50-μl reaction mixture containing 0.25 μM of each internal primer. The thermal cycling conditions were as before, except that an annealing temperature of 48°C was used.

Fifteen μl of nested PCR products of the HCMV and HHV-6 assays was analyzed by electrophoresis on 2% agarose gels (Promega, Madison, WI), whereas 3% gels were used for analyzing the 94-bp product of the HHV-7 PCR. A molecular size marker (ϕX174 DNA *Hae*III digest; Promega) was run on each gel.

The results of PCR amplification of HHV-7 DNA were confirmed by dot-blot hybridization with a T4 kinase-γ-^{32}P-labeled oligonucleotide probe 5′-ATC CTA ATG AAG GCT ACT TTG AAG TAC AAA TGT GC-3′ (probe sequence derived from genEMBL:Gb_vi:HHV-7ji, accession no. L 03525). The hybridisation was carried out using standard methods [Sambrook et al., 1989]. The hybridised membrane was washed twice at 60°C in 0.5 × SSC with 0.1% sodium dodecyl sulfate (SDS) buffer before autoradiography.

Serology

HHV-6 (AJ strain)-infected HSB-2 cells and HHV-7 (DC strain)-infected Sup-T1 cells were used in indirect immunofluorescence assays to detect HHV-6 and HHV-7 antibodies. The HHV-6 and HHV-7 strains were kindly provided by Professor R.S. Tedder (Department of Medical Microbiology, University College and Middlesex School of Medicine, London) and Dr. R.F. Jarrett (Leukaemia Research Fund Centre, University of Glasgow), respectively. Serology for capsid antigen (EBV VCA) IgG was done using B95-8 cells.

Clinical Definitions

The date of engraftment of the transplanted marrow was defined as the first day post-transplantation when the absolute neutrophil count was >500 cells/μl of blood. Clinical criteria used for staging of acute GVHD were based on the classification proposed by Glucksberg et al. [1974].

Criteria used for diagnosis of HCMV disease were adapted from Ljungmen and Griffiths [1993]. Briefly, HCMV pneumonitis was defined as clinical evidence of pneumonitis and/or hypoxemia and compatible radiographic findings in association with evidence of HCMV in bronchoalveolar lavage, as demonstrated by conventional culture or the detection of early antigen fluorescent foci (DEAFF) test. HCMV oesophagitis/colitis was defined by compatible clinical symptoms together with endoscopic evidence of inflammation and the detection of HCMV in biopsy specimens. HCMV hepatitis was defined as the presence of HCMV DNA in PBL together with otherwise unexplained elevation of hepatic parenchymal enzyme levels with or without confirmation by liver biopsy. HCMV leukopenia/thrombocytopenia was defined as unexplained leukopenia/thrombocytopenia together with HCMV DNA in PBL. When biopsies or autopsies were carried out, the finding of typical owl-eye inclusions with HCMV antigen demonstrated by immunocytochemical staining was considered as evidence of HCMV disease.

Patients with evidence of HCMV disease were treated with intravenous ganciclovir 5 mg/kg of body weight bid (dose adjusted if necessary for renal impairment) for 14 days and then given maintenance therapy 5 mg/kg of body weight daily or 3×/week for 4 weeks (or until day 100 post-transplantation), depending on the level of immunosuppression and the absolute neutrophil counts.

Statistical Analysis

Associations between groups of categorical data were tested using Fisher's exact test (two-tailed) or the chi-squared test for unpaired samples and using the McNemar's test for paired samples. The relationship between pre-engraftment viral DNA in PBL and time to engraftment was carried out by survival analysis, using the log-rank test.

RESULTS

Serology

Fifty-one (84%) of the 61 recipients were seropositive for HCMV. Six of these received autologous transplantation, 44 were matched with HCMV seropositive and 1 with HCMV seronegative donors. Among the 10 (16%) HCMV seronegative recipients, 4 were autologous transplants, 5 received an HCMV seropositive marrow, and the other a seronegative marrow. HHV-6 and HHV-7 serology was carried out for the last 17 patients and 8 donors. All were positive for HHV-6 and HHV-7 antibodies. The 30 healthy controls (age 21–45 years) selected for study were all seropositive for HCMV, HHV-6, and HHV-7 antibodies.

PCR Evaluation

PCR primers used to detect HCMV, HHV-6, and HHV-7 DNA, respectively, did not cross-amplify DNA from each other or that of other human herpesviruses,

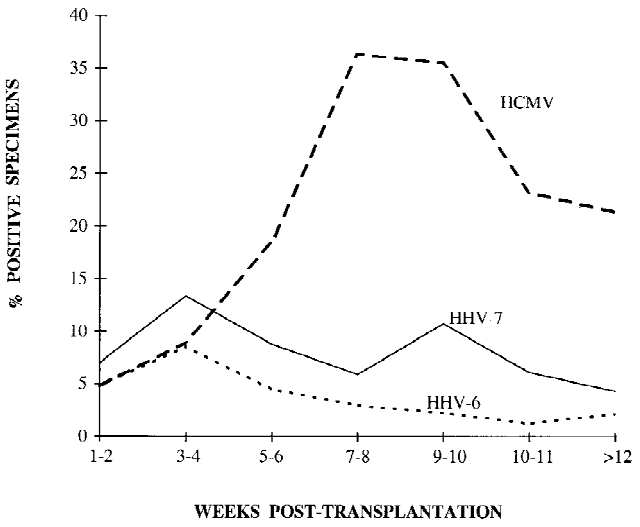

Fig. 1. HHV-6, HHV-7, and HCMV DNA in peripheral blood leukocytes post-transplantation.

including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), and Epstein-Barr virus (EBV) (data not shown). DNA from HHV-8 was not available for testing. The analytic sensitivity of the HCMV PCR had been determined previously to be 10–100 genome copies [Yuen et al., 1995]. The analytic sensitivities of HHV-6 and HHV-7 PCRs were estimated by using serial dilutions of the plasmid constructs pHV 6 and pHV 7 (kindly provided by Dr. P. Secchiero, National Institutes of Health, Bethesda) [Secchiero et al., 1995b] containing known copy numbers of the respective virus target DNA. Both PCRs detected approximately 5 copies of viral DNA in a 50-μl volume containing 5×10^6 PBL. The HHV-6 typing PCR produced the same analytic sensitivity as the screening PCR for variant B but was 10-fold more sensitive for HHV-6 variant A.

Profile of Viral DNA in Peripheral Blood Leukocytes After Bone Marrow Transplantation

None of the 30 PBL samples from the control group was positive for HCMV or HHV-6 DNA, and one (3%) was positive for HHV-7 DNA. Among the 563 blood samples collected from the 61 BMT recipients studied serially, HCMV, HHV-6, and HHV-7 DNA was detected in 130 (23%), 21 (4%), and 47 (8%) PBL samples, respectively. When analyzed by patient, 37 (61%) patients had HCMV DNA in PBL, 17 (28%) were HHV-6 DNA positive, and 32 (52%) were HHV-7 DNA positive. Of those patients who had detectable viral DNA in PBL, two or more DNA-positive specimens were found in 84% (HCMV), 12% (HHV-6), and 41% (HHV-7), respectively. Thus, in contrast to HCMV and HHV-7, HHV-6 DNA in PBL was usually transient. All 21 samples with HHV-6 DNA in PBL were found to be variant B in the variant typing PCR. The patterns of viral DNA positive PBL in relation to time post-transplant are shown in Figure 1, expressed as percentage of positive samples at different times post-transplant. HCMV and HHV-6 DNA in PBL showed a unimodal distribution with a peak at 7–10 weeks post-transplantation for HCMV and 3–4 weeks for HHV-6. In contrast, HHV-7 showed a bi-modal distribution with two peaks of positivity at 3–4 weeks and 9–10 weeks post-transplantation, respectively. Analysis based on percentage of patients positive, rather than percentage of samples positive, showed the same patterns (data not shown).

Clinical Characteristics of BMT Recipients Correlated With Viral DNA in Peripheral Blood Leukocytes

Patient characteristics. The characteristics of patients with or without HHV-6 or HHV-7 DNA in PBL were analyzed in relation to a number of clinical parameters and no significant associations were observed (Table I). Patients with allo-MUD transplants appeared to be more likely to have HHV-7 DNA in PBL, as compared to autologous transplants, although this trend was not statistically significant.

Other infections. The incidence of HCMV DNA in PBL overall was not correlated significantly with the presence or absence of either HHV-6 and HHV-7 DNA (Table I). The relationship of HHV-6 and HHV-7 DNA with HCMV disease is described separately (below). Bacterial, fungal, or viral infections other than HCMV also had no significant association with HHV-6 or HHV-7 DNA in PBL.

Progression of HCMV infection to HCMV disease. Thirty-seven of the 61 BMT recipients had HCMV DNA detectable in PBL, but only 13 of these patients had evidence of HCMV disease, including four with pneumonitis, three with hepatitis, four with leukopenia/thrombocytopenia, and two with colitis. In these 37 patients with detectable HCMV DNA, the association between HHV-6 or HHV-7 DNA in PBL and the risk of HCMV disease were studied. Each patient with HCMV disease ("case") was matched with a patient with HCMV DNA without disease ("control"). The criteria for selecting the matched controls were: HCMV DNA during the same post-transplant period, and the nearest sequential patient to fulfill the criteria. Thus, both "case" and matched "control" had HCMV PBL-DNA detected at similar times post-transplant, but only the "case" progressed to HCMV disease. Patients with HHV-6 or HHV-7 DNA detected in PBL during the 28-day period before the onset of HCMV disease ("cases") or the same observation period in the matched "controls" were identified for analysis (Table II). Of the 13 patients with HCMV DNA in PBL progressing to HCMV disease, 4 (31%) had HHV-7 DNA detected in PBL during the 28-day period prior to the onset of HCMV disease, whereas none of the matched "control group" was HHV-7 DNA positive during the corresponding period of observation. This association, though striking, did not achieve statistical significance possibly because of the small number of patients stud-

TABLE I. Clinical Characteristics of BMT Recipients Correlated With HHV-6 and HHV-7 DNA in PBL

Patient characteristics	HHV-6 DNA in PBL		HHV-7 DNA in PBL	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Total no. of patients (n = 61)	17 (28)	44 (72)	32 (52)	29 (48)
Diagnosis				
AML (n = 22)	7 (32)	15 (68)	12 (55)	10 (45)
CML (n = 17)	3 (18)	14 (82)	10 (59)	7 (41)
Others (n = 22)	7 (32)	15 (68)	10 (45)	12 (55)
BMT type				
Autologous (n = 11)	4 (36)	7 (64)	4 (36)	7 (64)
Allogeneic MRD (n = 43)	11 (26)	32 (74)	23 (53)	20 (47)
Allogeneic MUD (n = 7)	2 (29)	5 (71)	5 (71)	2 (29)
Total body/lymphoid irradiation (n = 33)	11 (33)	22 (67)	18 (55)	15 (45)
Patients with HCMV DNA in PBL (n = 37)	11 (30)	26 (70)	18 (49)	19 (51)

AML, acute myeloid leukaemia; CML, chronic myeloid leukemia; MRD, matched related donor; MUD, matched unrelated donor. No significant association ($P > 0.05$) was found by Fisher's exact test or by χ^2 test.

TABLE II. Association of HHV-6 and HHV-7 DNA in PBL With HCMV Disease and Asymptomatic HCMV Infection

Viral DNA in PBL[a]	HCMV disease (n = 13)	Asymptomatic HCMV infection[b] (n = 13)	P[c]
HCMV + HHV-6	2 (15%)	1 (8%)	1.0
HCMV + HHV-7	4 (31%)	0 (0%)	0.13
HCMV + HHV-6 + HHV-7	1 (8%)	0 (0%)	1.0

[a]Viral DNA in peripheral blood leukocytes (PBL) detected during the 28 days prior to onset of HCMV disease.
[b]Thirteen matched controls with HCMV DNA in PBL without progression to HCMV disease. Criteria for selection in text.
[c]McNemar's test.

ied. There was no comparable trend between HHV-6 and HCMV disease.

Marrow engraftment. Two of the 61 patients studied failed to engraft. The first patient received an allogeneic transplant for aplastic anemia and HHV-7 DNA was first detected in PBL on day 38. HHV-6 DNA was not detected throughout the observation period. A repeated transplant also failed and the patient died subsequently. The second patient was a 33-year-old man who received matched unrelated marrow for CML. Although no viral DNA was detectable in PBL to any of the viruses, HHV-6 DNA was detected on the plasma sample collected on day 39 post-transplantation. This was preceded by high fever and was associated with a maculopapular rash on the trunk and limbs. A skin biopsy and the absence of engraftment excluded GVHD as a cause of the skin rash. Treatment with G-CSF and GM-CSF failed to induce engraftment. A second peripheral blood stem cell infusion was done after reconditioning with high-dose steroids and OKT3, after which engraftment occurred.

In patients with and without HHV-6 and HHV-7 DNA detectable in PBL prior to engraftment, the time taken for neutrophil engraftment was analyzed by survival analysis. The two patients who failed to engraft were censored at day 40 post-transplant for the purposes of this analysis. Allogenic matched related donor transplant recipients in whom HHV-7 DNA was detectable in PBL before engraftment took a significantly longer time to engraft (mean 28.8 vs 19.8 days; log-rank test: $P = 0.01$). A comparable effect was not seen for patients with HHV-6 DNA in PBL (Table III). The numbers of patients with autologous transplants and matched unrelated donor grafts were too small for statistical analysis.

Graft-versus-host disease. Forty-seven percent (20/43) of the allogeneic and 71% (5/7) of the matched unrelated graft recipients developed GVHD during the first 90 days post-transplantation. Two had hyperacute GVHD occurring during the first week post-transplantation, the other 23 had acute GVHD with clinical onset ranging from days 14–44 post-transplantation (mean, day 27). Among the 25 patients with acute GVHD, 9 (36%) had HHV-6 DNA detected in PBL during the first 90 days post-BMT, and in 4 of them, the viral DNA was positive before the clinical onset of disease. In 11 (44%) patients with acute GVHD, HHV-7 DNA was detected in PBL within the first 90 days post-BMT, and in 4 of them, this occurred before the clinical onset of disease. To analyze the association between HHV-6 and HHV-7 with acute GVHD, each patient with acute GVHD ("case") was matched with a "control" based on the following criteria: (1) no clinical evidence of GVHD; (2) whether matched related or matched unrelated transplant; and (3) the nearest sequential patient to fulfill the criteria. Viral DNA prior to GVHD was defined as DNA detected at any time before clinical onset of GVHD, the same observation period being applied to the matched control. Viral DNA subsequent to GVHD was defined as DNA detected anytime during the course of disease and up to 14 days after clinical recovery (to account for the tailing effect of anti-GVHD therapy). The same observation period was applied in the matched controls. HHV-6 DNA was detected in PBL prior to "GVHD" in 4 (16%) of 25 and 2 (8%) of 25 of the GVHD and control groups respectively (McNemar's test; $P = 0.61$). Similarly, HHV-7 DNA was detected in PBL in 16% and 20% of the GVHD and control groups ($P = 1.0$).

The prevalence of HHV-6 DNA in PBL during or up to 14 days after "GVHD" was 5/25 (20%) and 1/25 (4%) in the GVHD and control groups, respectively ($P =$

TABLE III. Association Between HHV-6 and HHV-7 DNA in PBL and Time to Neutrophil Engraftment

Viral DNA in PBL	Mean time in days (± SD) taken for neutrophil engraftment		
	Autologous	Allo-MRD	Allo-MUD
HHV-6 DNA positive	NA (n = 0)	22.7 ± 6.8 (n = 3)	30[a,b] ± 8.7 (n = 3)
HHV-6 DNAemia negative	15 ± 4.3 (n = 11)	20.8[a] ± 6.5 (n = 40)	23.8 ± 2.2 (n = 4)
Log-rank test	NA	$P = 0.73$	NA
HHV-7 DNAemia positive	21 ± NA (n = 1)	28.8[a] ± 8.5 (n = 5)	26 ± NA (n = 1)
HHV-7 DNAemia negative	15 ± 6.1 (n = 10)	19.8 ± 5.5 (n = 38)	26.5[a] ± 6.8 (n = 6)
Log-rank test	NA	$P = 0.01$	NA

[a]One patient failed to engraft. Data censored at 40 days for this analysis.
[b]HHV-6 positive only from plasma in one patient who failed to engraft.
NA, not applicable (sample size too small); SD, standard deviation.

0.22). The data for HHV-7 DNA for the two groups were 3/25 (12%) and 8/25 (32%), respectively ($P = 0.23$).

Effect of Antiviral Therapy on Viral DNA in Peripheral Blood Leukocytes

Prophylactic acyclovir, 200 mg tid PO, or 5 mg/kg of body weight tid IV, was given to all allografts and autografts during the first 30 days post-BMT. The antiviral prophylactic regimen for matched unrelated transplant was intravenous acyclovir 10 mg/kg of body weight tid until engraftment, followed by intravenous ganciclovir 5 mg/kg of body weight 3×/week until 120 days post-BMT. Patients with two or more consecutive PBL positive for HCMV DNA or with a single DNA positive while on severe immunosuppression were considered for pre-emptive therapy with intravenous ganciclovir (5 mg/kg of body weight daily). The DNA positive rates of specimens collected after patients had been on 2 or more days of acyclovir or ganciclovir therapy (to allow attainment of stable blood levels of the drug) are shown in Table IV. Positive rates of HHV-6 DNA in PBL specimens collected when the patients were on prophylactic or treatment dosage regimens of acyclovir were similar to those collected when not on acyclovir. However, none (0/16) of the specimens collected from patients on "treatment dose" of acyclovir (10 mg/kg of body weight tid IV) had HHV-7 DNA in PBL, in contrast to 11% (14/127) positivity in the specimens from patients on a "prophylactic dose" of acyclovir. This difference was not statistically significant ($P = 0.38$).

None of 17 specimens collected while patients were receiving ganciclovir treatment (5 mg/kg of body weight 1×/day or 2×/day) were positive for either HHV-6 or HHV-7 DNA, whereas the positive rates of specimens collected during 3×/week ganciclovir were similar to those without ganciclovir administration (Table IV). Again, the differences were not statistically significant.

HHV-6 and HHV-7 DNA in Plasma Specimens

A total of 179 plasma samples collected from 17 patients were available for HHV-6 and HHV-7 PCR. The QIAamp Blood Kit (QIAGEN, Chatsworth, CA) used according to the manufacturer's instructions was compared with standard proteinase K digestion, phenol-chloroform extraction, followed by ethanol precipitation on 200 μl plasma samples spiked with tissue culture-grown virus. The QIAamp Blood Kit gave superior results (data not shown) and has been used for viral DNA extraction from the plasma samples in this study. Only three plasma samples were positive for virus: two positive for HHV-6 DNA and one positive for HHV-7 DNA. The viral DNA was also detected in the corresponding leukocyte lysate in two patients. The third patient failed to engraft and had a leukocyte count of $<0.1 \times 10^9$ cells/L, probably explaining why the PBL-DNA was negative, although plasma contained HHV-6 DNA.

In two patients, the plasma viremia (HHV-7 and HHV-6, respectively), correlated with neurological dysfunction and an encephalitis-like illness. The first patient was a 15-year-old boy (UPN 258), who received a matched related allograft for acute lymphoblastic leukemia. A surveillance blood collected on day 160 post-transplantation was positive for HHV-7 DNA in both plasma and leukocyte fractions. Five days later, he developed a rapid and profound alteration of cognitive function associated with a skin rash. The cerebrospinal fluid (CSF) had 15 white blood cells per cubic milliliter (WBC/ml[3]) (85% lymphocytes), elevated total CSF protein of 1.6 g/L (normal range 0.15–0.45 g/L) and glucose of 4.8 mmol/L (76% of blood level). Magnetic resonance imaging (MRI) revealed enhancement over the left frontal lobe and mild cerebral atrophy. Microbiological investigation of the CSF was negative, including PCR for HCMV, HSV, and *Mycobacterium tuberculosis*. A skin biopsy done 4 days after the onset of the neurological disease revealed nonspecific inflammation with no evidence of GVHD. He had two episodes of generalized tonic-clonic convulsions but eventually made a recovery from the encephalitic illness. The antibody titres to HHV-7, HHV-6, and EBV are shown in Table V. The HHV-7 results were available only in retrospect, and the CSF was not available for HHV-7 or HHV-6 PCR.

The second patient, a 26-year-old man (UPN 265) received an HLA-A antigen mismatched sibling transplant for chronic myeloid leukemia. HHV-6 DNA was detected in both PBL and plasma on day 24 associated with high fever. He had an episode of a confusional

TABLE IV. Percentage of PBL Specimens Positive for HHV-6 and HHV-7 DNA While on Antiviral Therapy*

Viral DNA in PBL	Acyclovir[a]			Ganciclovir[b]		
	Not treated (n = 420) (%)	Prophylaxis (n = 127) (%)	Treatment (n = 16) (%)	Not treated (n = 492) (%)	3 times/wk (n = 54) (%)	12–24 hourly (n = 17) (%)
HHV-6	3	6	6	4	4	0
HHV-7	8	11	0	9	7	0

*Only specimens collected after 48 hr of commencement of antiviral agent are included in the treatment or prophylaxis groups.
[a]Acyclovir prophylaxis: 200 mg tid PO or 5 mg/kg of body weight tid IV; treatment: 800 mg 5×/day PO or 10 mg/kg of body weight tid IV.
[b]Ganciclovir: 5 mg/kg of body weight per dose intravenously.
N, number of specimens.

TABLE V. HHV-6 and HHV-7 Serological Results on Two Patients With Neurological Disease

Patient	Days post-transplant	HHV-6 IgG titer	HHV-7 IgG titer	EBV VCA IgG titer
UPN 258	108	1/200	1/50	1/640
	160 plasma HHV-7 DNA pos	ND	ND	ND
	187	1/1,600	1/400	1/640
	234	1/400	1/200	1/1,280
UPN 265	10	1/100	<1/50	1/1,280
	24 plasma HHV-6 DNA pos	1/50	ND	ND
	50	1/400	1/100	1/256
	62	1/25	1/100	1/1,280

ND, not done.

state that was attributed to Cyclosporin A toxicity, which initially improved on Cyclosporin A withdrawal. But the confusional state recurred and MRI showed a bilateral increase of signal over the hippocampus area, compatible with edema or demyelination. He was treated empirically with acyclovir and later with foscarnet, with no response. Because of the low platelet count (15×10^9 cells/L) a lumbar puncture was not done until 2 weeks after the onset of neurological symptoms, by which time the CSF was unremarkable. No pathogens were detectable in the CSF (including negative PCR results for HCMV, HSV, and *Mycobacterium tuberculosis*). His serological profile is shown in Table V. As with the previous patient, the HHV-6 results were only available in retrospect, and no CSF remained for HHV-6 or HHV-7 PCR.

In the third patient, HHV-6 was detected in plasma on day 39 after a MUD transplant. This was associated with fever, rash, and failure to engraft and his clinical course has been described above (see section on engraftment).

DISCUSSION

The clinical significance of HHV-6 or HHV-7 DNA detection in PBL by PCR in an individual patient is difficult to establish because of the persistence of these viruses in peripheral blood mononuclear cells. The presence of viral DNA in healthy controls may represent asymptomatic reactivation, persistence of latent viral genome or persistent low-level viral replication. Although attribution of clinical significance to detection of HHV-6 or HHV-7 DNA in PBL in an individual patient may prove difficult, overall patterns in a patient population and their correlation with clinical outcome may provide useful information. The detection rate of HHV-6 and HHV-7 DNA in PBL by PCR varies with the sensitivity of the PCR method used and the leukocyte DNA concentration added to the PCR reaction. It is therefore difficult to compare levels of positivity in different studies. In this study, DNA from $2.5–5 \times 10^3$ cells from healthy controls or BMT recipients were added to each PCR reaction, which is a lower input of leukocyte DNA as compared to some other studies and possibly explains the lower PBL-DNA positive rate in healthy controls (0% and 3% for HHV-6 and HHV-7, respectively). The lower sensitivity in our study was a deliberate attempt to discriminate between persistent viral DNA in "healthy" carriers of the virus and patients with active viral replication or disease where the viral load is likely to be higher, as has been observed in patients with HCMV disease [Fox et al., 1995].

When immunocompromised patients have HCMV DNA in PBL, they often remain positive over consecutive weeks, unless given appropriate antiviral treatment. In contrast, most HHV-6 DNA episodes were single positive results. This is in agreement with a previous study on renal transplant recipients [Osman et al., 1996].

The peak of HHV-6 DNA positivity in PBL was found 3–4 weeks post-BMT, was earlier than that usually seen for HCMV, and resembled the pattern seen with herpes simplex virus [Engelhard et al., 1986]. Other studies employing PCR [Drobyski et al., 1993b; Wang et al., 1996] or virus isolation methods [Yoshikawa et al., 1991] have made similar observations for HHV-6. In contrast, we found HHV-7 PBL-DNA positivity has a bimodal distribution during the post-transplant pe-

riod with peaks at 3–4 weeks and 9–10 weeks post-transplantation. The first peak of HHV-7 preceeded that of HCMV.

Our finding that the HHV-6 DNA found in PBL is predominantly variant B virus is in agreement with other studies in marrow transplant [Drobyski et al., 1994; Wilborn et al., 1994; Wang et al., 1996] and oncology patients [Lyall et al., 1995]. The results showed that neither the use of total body/lymphoid irradiation nor the underlying disease pathology leading to transplant was associated with differences in the occurrence of HHV-6 or HHV-7 DNA in PBL during the post-transplant period. However, there was a trend to an increase in HHV-7 DNA in patients receiving allo-MUD, as compared to autologous transplants, which is likely to be due to the more intense level of immunosuppression in the former group of patients. HCMV infection is previously known to be a risk factor for opportunistic infections, but our data did not show any significant associations between HHV-6 and HHV-7 DNA in PBL and bacterial, fungal, or viral disease (excluding HCMV).

Drobyski et al. [1993a] reported a myelosuppressive effect of HHV-6 in BMT recipients. Both of our patients who failed to engraft were associated with either HHV-6 or HHV-7. One patient had detectable HHV-6 DNA in plasma in association with fever and skin rash (not due to GVHD), failed to engraft, and had to be given a second transplant. Although the graft finally took hold, he subsequently had a stormy course with severe GVHD and HCMV disease and succumbed. In the second patient, HHV-7 DNA was detected in PBL on day 38; this patient failed to engraft, and a repeat transplant failed as well. Further analysis on the whole patient group suggested that HHV-7 DNA in PBL prior to engraftment may be associated with a delay in engraftment. Some of the previous studies where HHV-6 antigens were demonstrated in the bone marrow of patients with myelosuppression used rabbit polyclonal antibody reagents [Knox and Carrigan, 1996] and cross-reactions with HHV-7 cannot be excluded. The possibility that some of the reported cases of myelosuppression attributed to HHV-6 by immunohistological techniques may in fact be due to HHV-7 deserves consideration. Only one other study [Wang et al., 1996] has specifically investigated the relationship between HHV-7 and engraftment after marrow transplantation, but they failed to find an association. However, these investigators did not differentiate matched unrelated donor allografts from related donor allografts, a factor that may confound studies on engraftment. In vitro data [Furukawa et al., 1994] suggest that HHV-7 may be immunosuppressive and immunomodulatory. Further study on its effect on the bone marrow is warranted.

The etiologic role of HHV-6 in exanthem subitum has been well established. However, its association with skin rash in immunocompromised hosts, particularly in BMT recipients, has not been well defined. Yoshikawa et al. [1991] reported an increase in incidence of HHV-6 DNA in PBL in BMT recipients who developed a skin rash with a clinical diagnosis of acute GVHD. Appleton et al. [1995] showed an association between the increased severity of GVHD and the detection of HHV-6 DNA in post-BMT biopsy tissue obtained prior to, or concomitant with, the onset of GVHD and suggested that HHV-6 preceded GVHD, rather than reactivated as a consequence of GVHD. We attempted to analyze the association between these viruses and GVHD by observing PBL-DNA positivity in patients with GVHD ("cases") and matched "controls." No significant association was found between HHV-6 or HHV-7 DNA in PBL preceding the onset of clinical GVHD, a finding similar to that reported by others [Wang et al., 1996; Appleton et al., 1995]. However, Appleton and colleagues [1995] found an association between HHV-6 DNA and GVHD when serial biopsy specimens were examined. In the present study, we do not have similar data for comparison.

On the other hand, our data show a higher rate of HHV-6 DNA following GVHD (20%), as compared with controls during the matched observation period (4%). Although not statistically significant, this raises the possibility that GVHD itself or associated immunosuppressive therapy is associated with increased HHV-6 reactivation.

The impact of HCMV on the outcome of marrow graft recipients has been well characterized. However, only some patients with HCMV viremia develop overt disease [Meyers et al., 1990]. This finding suggests that other factors may be relevant in the progression of HCMV infection to HCMV disease. We analyzed the role of HHV-6 and HHV-7 in this respect by observing HHV-6 or HHV-7 DNA in PBL occurring during the 28 days before the clinical onset of HCMV disease. This period was chosen to allow time for indirect mechanisms such as immunomodulation, as well as for direct viral effects (cytopathic or immunopathological) on target organs to play a role in the pathogenesis of HCMV disease. Those patients with HCMV disease were more frequently associated with preceding HHV-7 DNA (31%) than were those with asymptomatic HCMV infection (0%). This trend did not achieve statistical significance ($P = 0.13$) probably due to the small numbers of patients studied, but does support previous observations in renal allograft recipients [Osman et al., 1996]. Further, a significant association between HHV-6 seroconversion and HCMV disease has been observed in liver transplant recipients [Dockrell et al., 1996]. There is extensive serological cross-reaction between HHV-6 and HHV-7 [Black et al., 1996]. It is therefore possible that the serological response to HHV-6 seen in the liver transplant recipients could reflect either HHV-6 or HHV-7 infection. If so, their data also support our own observations that HHV-7 is a co-factor for HCMV disease. The immunomodulatory potential of HHV-7 has previously been described in vitro, and may be relevant in this regard [Furukawa et al., 1994]. Transactivation of the HCMV immediate early gene by the HHV-6 ZVH14 ORF has been documented [Razzaque and

Jones, 1995]. Further, HHV-7 has been shown to reactivate latent HHV-6 in vitro [Katsafanas et al., 1996]. If HHV-7 similarly induces the reactivation of HCMV, this may result in HHV-7 acting as a cofactor in HCMV disease. An alternative explanation for these observations however, is that an independent confounding factor precipitates both HCMV disease and HHV-7 reactivation.

Our observation on effects of antiviral agents on HHV-6 DNA in PBL was in keeping with the known in vitro findings on the susceptibility of HHV-6 to antivirals [Russler et al., 1989; Burns et al., 1990]. The antiviral susceptibility of HHV-7 has not been well characterized. Although the numbers of patients were too small to demonstrate statistical significance, the trends observed suggest that at full therapeutic dosage, both acyclovir and ganciclovir may suppress HHV-7 activity in vivo. However, it is not possible to conclude that HHV-7 disease is treatable with these antiviral agents. On the other hand, prophylactic or suppressive dosage regimens of acyclovir or ganciclovir had no obvious impact on either HHV-6 or HHV-7. If HHV-6 and/or HHV-7 are shown to be important pathogens after BMT, information on the effect of antiviral agents on viral DNA in vivo would be useful in guiding trials of pre-emptive therapy, an approach that has already proved be successful in the prevention of HCMV disease.

HHV-6 and HHV-7 DNA may sometimes be detected in PBL of "healthy" controls and cannot always be correlated with disease in the immunocompromised patient. However HHV-6 and HHV-7 DNA is not detectable in the plasma of healthy controls and are less frequent during the post-BMT period—only 2 (1.1%) and 1 (0.6%) of 179 plasma samples being positive for HHV-6 and HHV-7, respectively. Viral DNA in plasma possibly indicates significant levels of viral activity and has been shown to predict HCMV disease better than HCMV DNA in PBL [Spector et al., 1992]. In addition, viral DNA in plasma may be the only option for detecting viral activity during the pre-engraftment period or in patients who fail to engraft. All three patients with either HHV-6 or HHV-7 DNA in plasma had otherwise unexplained clinical diseases associated in time with the positive result; one had skin rash, fever, and failure of engraftment associated with HHV-6 and has been discussed above. The other two patients had HHV-7 and HHV-6 DNA, respectively, detected in the plasma (as well as in PBL) and were associated with neurological disease. In these two patients, the plasma viremia was associated with a rise in IgG antibody titer to both HHV-6 and HHV-7. Since HHV-6 and HHV-7 are closely related viruses, it is not surprising that reactivation of either virus in previously seropositive patients gives rise to rising antibody titers to both. Since serology in the post-transplant period can be difficult to interpret, we also determined the antibody titers to EBV (also carried out by immunofluorescence techniques) to serve as a "control." The rise in HHV-6 and HHV-7 IgG titers was not associated with a corresponding rise in the EBV titers at the same time, supporting the contention that the HHV-6 and HHV-7 serological responses were likely to be due to reactivation of one or both of these viruses.

Two patients with primary HHV-7 infection leading to exanthem subitum and central nervous system (CNS) manifestations have recently been reported [Torigoe et al., 1996], but there is no previous report of viral reactivation associated with encephalitic disease. The only other disease association of HHV-7 has been as a possible cofactor in HCMV disease [Osman et al., 1996]. While it is unfortunate that we were unable to investigate the CSF for evidence of HHV-7 DNA, the detection of viral DNA in plasma together with the serological response is suggestive of a disease association. The fact that biopsy evidence suggested that the skin rash was unlikely to be due to GVHD supports the contention that the illness was HHV-7 related.

Immuno-histological evidence of HHV-6 in the brain has been documented previously in fatal encephalitis following BMT [Drobyski et al., 1994], and this virus has been incriminated as a possible cause of focal encephalitis in immunocompetent patients on the basis of HHV-6 DNA detection in the CSF [McCullers et al., 1995]. The patient reported here may be another instance of HHV-6 neurological disease. However, ascribing etiology to events early in the post-transplant period is particularly difficult. This patient received multiple blood products early in the post-transplant period, making the serological data difficult to interpret.

We report an analysis of the activity of the three human herpesviruses—HCMV, HHV-6, and HHV-7—in a cohort of BMT recipients. Our data show that each virus has a distinct but overlapping pattern of reactivation and that there may be interactions in relation to disease progression. The value of monitoring viral DNA in plasma as a noninvasive indicator of high levels of viral activity in immunocompromised individuals is highlighted. The possible association of HHV-7 with a focal encephalitic disease and delayed engraftment is reported. Further studies on immunocompromised patients using quantitative PCR combined with viral antigen detection in target tissues may confirm the clinical significance of HHV-7.

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